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High Incidence of T-Cell Tumors in E2A-Null Mice and E2A/Id1 Double-Knockout Mice

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The basic-helix-loop-helix (bHLH) proteins encoded by the E2A gene are broadly expressed transcription regulators which function through binding to the E-box enhancer sequences. The DNA binding activities of E2A proteins are directly inhibited upon dimerization with the Id1 gene product. It has been shown that disruption of the E2A gene leads to a complete block in B-lymphocyte development and a high frequency of neonatal death. We report here that nearly half of the surviving E2A-null mice develop acute T-cell lymphoma between 3 to 10 months of age. We further show that disruption of the Id1 gene improves the chance of postnatal survival of E2A-null mice, indicating that Id1 is a canonical negative regulator of E2A and that the unbalanced ratio of E2A to Id1 may contribute to the postnatal death of the E2A-null mice. However, the E2A/Id1 double-knockout mice still develop T-cell tumors once they reach the age of 3 months. This result suggests that E2A may be essential for maintaining the homeostasis of T lymphocytes during their constant renewal in adult life.

Helix-loop-helix (HLH) proteins belong to a family of transcription factors which play important roles in cell fate determination and cellular differentiation (for reviews, see references 29 and 42). They contain a highly conserved sequence motif known as the basic helix-loop-helix (bHLH) domain. The bHLH motif can be further divided into two subdomains, the HLH domain and an adjacent basic region. The HLH domain, consisting of two amphipathic α-helices separated by a loop of amino acid sequence of variable length, is responsible for the dimerization between HLH proteins. The basic region, consisting of a cluster of 16 to 20 amino acids rich in lysine and arginine residues that lie just N terminal to the first helix, mediates high-affinity, sequence-specific DNA binding. Most bHLH proteins recognize a common CANNTG core sequence known as the E box (27). Many bHLH proteins are expressed in a temporally and spatially restricted manner. Extensive studies of these proteins have shown that they are required for tissue-specific gene expression. For example, the MyoD family is required for muscle-specific gene expression (42), the *achaete scute* family is involved in neuronal differentiation (17), and SCL1 is essential for early hematopoiesis (36).

The tissue-specific bHLH proteins often bind DNA as heterodimers with a set of ubiquitously expressed bHLH proteins known as E proteins. The E-protein genes are expressed in a non-tissue-specific manner. Heterodimers between E proteins and tissue-specific bHLH proteins are usually more effective than homodimers in up-regulating the transcription of target genes (25). Three E-protein genes, namely, E2A, E2.2, and HEB, have been identified in mammals. The human E2A gene encodes two bHLH proteins, E12 and E47, through differential splicing of two bHLH-encoding exons (27). They were initially identified in B cells as immunoglobulin (Ig) enhancer binding proteins but were subsequently found to be present in most cell types. However, the DNA binding activity of E2A homodimers seems to be absent in most cell types except B cells (35). It has been shown that posttranslational modifications, such as the formation of an intermolecular disulfide bond and phosphorylation, modulate the homodimerization potential of the E2A proteins in a cell-type-specific manner (4, 37). The importance of E2A in B-cell development was further supported by gene-targeting experiments that demonstrated a complete lack of B cells in E2A-null mice (1, 46). Additionally, overexpression of E47 is sufficient to activate the chromosomal Ig heavy chain and terminal deoxynucleotidyltransferase loci in non-B cells (7, 34). Given this evidence and the fact that so far no B-cell-specific bHLH gene product has been found, it is likely that the E2A homodimer is the functional transcription factor which is required for B-cell lineage development.

In addition to their roles in B-cell formation, E2A proteins have been shown to have growth-inhibitory properties: when overexpressed in fibroblasts, these proteins can block cell cycle progression near the G_1-S boundary (30). A role for E2A in growth regulation is further supported by the study of human B-cell acute lymphocytic leukemia (B-ALL). The E2A locus is involved in two types of translocation associated with human lymphoid leukemia (16, 22, 28). These translocations, which account for roughly 25% of B-ALL, link the E2A gene to one of two novel genes, one (*pbx1*) encoding a homeodomain protein and the other (*hlf*) encoding a b-ZIP (leucine zipper) transcription factor. Both types of translocation produce fusion proteins which splice the transactivation domain of E2A to the DNA binding domains of the fusion partners (16, 22). Work carried out in several laboratories has shown that the fusion proteins are oncogenic both in tissue culture cells and in transgenic mice (9, 21, 44). Together, these studies indicate that E2A proteins may be involved in cell growth-regulatory pathways which directly control the cell fate decision between proliferation and differentiation. This notion is further substantiated by the finding that disruption of the E2A gene induces thymic T-cell tumors (see below).

E2.2 and HEB gene products are structurally and function-

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ally related to the E2A gene products, and all are ubiquitously expressed (13, 15). Genetic studies revealed that E2.2 and HEB are also involved in B-cell development (45, 46). While E2A-null mice make no pro-B cells, mice homozygous for the E2.2 and HEB mutations can make B cells, but with reduced numbers of pro-B cells. A reduction in pro-B cells was also observed in mice transheterozygous for the E2A, E2.2 or HEB mutations, strongly suggesting that the functions of the three E proteins are related (43).

In addition to having a role in B-cell development, each of the three E-protein genes is required for postnatal development. E2A-null mice are born at a lower frequency than wild-type mice, and most of them die within the first 2 weeks after birth, with evidence of severe growth retardation. Very few mice (about 5%) are able to survive to adulthood. E2.2-null mice are born at an extremely low frequency and have never survived more than a week after birth. Mice homozygous for the HEB mutation are born at the expected frequency, but most die within the first 2 weeks after birth. The cause of death remains unknown but it appears to be independent of the B-cell and T-cell phenotypes, since both cell types are not essential for mice to survive in a sterile environment. The postnatal lethality phenotype is also observed in every transheterozygous combination among the three mutations, again suggesting that the three E-protein genes function in related pathways.

It is established that E proteins are capable of interacting with the products of the Id gene family. The Id proteins, which are encoded by four separate genes (Id1, -2, -3, and -4), lack the basic region essential for DNA binding and thus function as dominant negative regulators of E2A and other bHLH proteins (5, 8, 10, 32, 39). The Id1 gene is highly expressed in undifferentiated, cycling cells and tumor cell lines, and its expression level decreases dramatically when cells undergo terminal differentiation (5). Overexpression of Id1 blocks the cellular differentiation program in a variety of cell culture systems and in transgenic mice (5, 19, 24, 26, 38). Several lines of evidence also suggest that Id proteins play a role in G_0-S phase transition of the cell cycle. The stimulation of quiescent fibroblasts with serum or growth factors induces the transcription of the Id genes (2, 5, 8, 11). Inhibiting Id protein synthesis by antisense oligonucleotides and microinjection of anti-Id1 antibodies prevents the reentry of arrested cells into the cell cycle (2, 12, 30). This effect of Id proteins in growth regulation is probably mediated by E proteins. It has been shown that >90% of E2A is associated with Id1 in proliferating myoblasts (19). In addition, overexpression of Id1 can overcome the growth-inhibitory effect of E2A, suggesting that the balance between E2A and Id1 is critical in regulating cell proliferation (29).

In this study, to test the hypothesis that deregulated activity of the growth-promoting Id genes is responsible for the phenotypes observed in E2A-null mice, we have generated Id1 knockout mice by gene targeting and crossed them with E2A-null mice to generate E2A/Id1 double-knockout mice. These mice survived at a significantly higher frequency than E2A-null mice, clearly indicating a genetic interaction between E2A and Id1 in the regulation of postnatal development. In addition, we observed a high incidence of T-cell tumors both in E2A-null mice and in E2A/Id1 double-knockout mice between the ages of 3 to 10 months, identifying a previously unknown function of E2A in the maintenance of homeostasis of T lymphocytes.

MATERIALS AND METHODS

Targeted disruption of the Id1 gene. The linearized targeting vector was transfected into an embryonic stem cell line CJ7 (40). G418-resistant clones were screened by PCR and Southern blotting. Three embryonic stem cell clones that had undergone homologous recombination with the targeting vector were injected into blastocysts. Heterozygous offspring of the germ line-transmitting chimeras were bred into a pure 129sv background and a mixed background (C57BL/6 and 129sv) to obtain homozygous mice.

Mouse genotyping. The genotype of each mouse was determined by PCR analysis (45) of tail or toe DNA. Each locus requires three oligonucleotide primers in one PCR which generates two distinct products representing the wild-type and mutant alleles. The primer set for the Id1 locus includes prK4 (5′GGTTGCTTTTGAACGTTCTGAACC; common primer), pr22 (5′-CCTCA GCGACACAAGATGCGATCG; wild-type-specific primer), and prPGK (5′GC ACGAGACTAGTGAGACGTG; mutant-specific primer). The PCR gives rise to an 800-bp product and a 500-bp product for the wild-type and mutant alleles, respectively. The primer set for the E2A locus includes YZ-104 (5′ATGTGTG GTGGCCCACACTTGT3′; common primer), YZ-164 (5′AAGAACGAGGCC TTCCGTGTC3′; wild type-specific primer), and YZ-29 (5′TCGCAGCGCATC GCCTTCTA3′; mutant-specific primer). The PCR gives rise to a 1.7-kb fragment and a 2.0-kb fragment for the wild-type and mutant alleles, respectively.

Fluorescence-activated cell sorting (FACS) analysis. Thymocytes, spleen cells, and peripheral blood cells were resuspended in phosphate-buffered saline supplemented with 5% bovine calf serum. Cells were stained with phycoerythrin (PE)- and/or fluorescein isothiocyanate (FITC)-conjugated monoclonal antibodies (purchased from Sigma and Pharmingen). 7-Amino-actinomycin D was included in the staining to label the dead cells (45). Data were collected on a FACScan flow cytometer and analyzed with the CellQuest and LysisII programs (Becton Dickinson).

Southern and Northern analyses. Genomic DNA was purified from mouse tail tips and separated on a 1% agarose gel after digestion. Southern analysis was performed by using a *Sal*I-*Bam*HI fragment from Φ811 (a gift from K. Winter) that encompasses the Id1 locus. Total RNA was prepared from primary embryonic fibroblasts (PEF cells) derived from $Id1^{+/-}$ or $Id1^{-/-}$ mice. RNA (10 μg) was used for Northern hybridization with the following probes: Id1, a PCR-synthesized probe containing nucleotides 265 to 383 of cDNA; Id3, a PCR-generated probe corresponding to nucleotides 365 to 635 of Id3 cDNA (18); and GAPDH (glyceraldehyde-3-phosphate dehydrogenase), a 1-kb *Pst*I fragment of pR-GAPDH (41).

Histology. Tissue samples were preserved in phosphate-buffered saline–buffered 10% formalin, dehydrated, and embedded in paraffin. Six-micrometer sections were stained with hematoxylin and eosin and examined by light microscopy.

RESULTS

E2A-null mice developed T-cell tumors between the ages of 3 to 10 months. E2A knockout mice were recently generated by deletion of the bHLH domains of both E12 and E47 proteins through homologous recombination (1, 46). These mice produce no B cells and die postnatally at a high frequency. For unknown reasons, a small fraction of E2A-null mice can live for up to 10 months. Through 2 years of breeding in the mixed background of 129/sv and C57BL/6, we have thus far obtained and analyzed 13 adult E2A-null mice. Six of these mice developed acute T-cell tumors, three were sick due to incidental bacterial infections, and the rest were normal at the time of analysis. During the same breeding period, only one E2A heterozygous mouse developed T-cell leukemia after reaching the age of 2 years. The development of T-cell tumors in adult E2A-null mice was apparently unrelated to the events that led to neonatal death. The high rate of neonatal death was correlated with severe growth retardation, and no T-cell tumors were found at this young age.

The E2A knockout mice which had developed T-cell tumors were hunchbacked, skinny, and physically weak. Upon dissection, an enlarged thymus and/or tumor mass was readily visible inside the rib cage. FACS analysis with T-cell markers demonstrated that the tumors were derived from immature thymocytes (Fig. 1). In most cases, these tumor cells were also detected in the blood, as well as lymphoid and nonlymphoid organs, indicating the malignant nature of these tumors. Thus far, three types of T-cell tumors including $CD4^+$ $CD8^+$, $CD4^{low}$ $CD8^+$, and $CD4^-$ $CD8^+$ have been observed in separate animals (Table 1), and these tumor cells express either a low level of or no surface T-cell receptors. The tumor cells

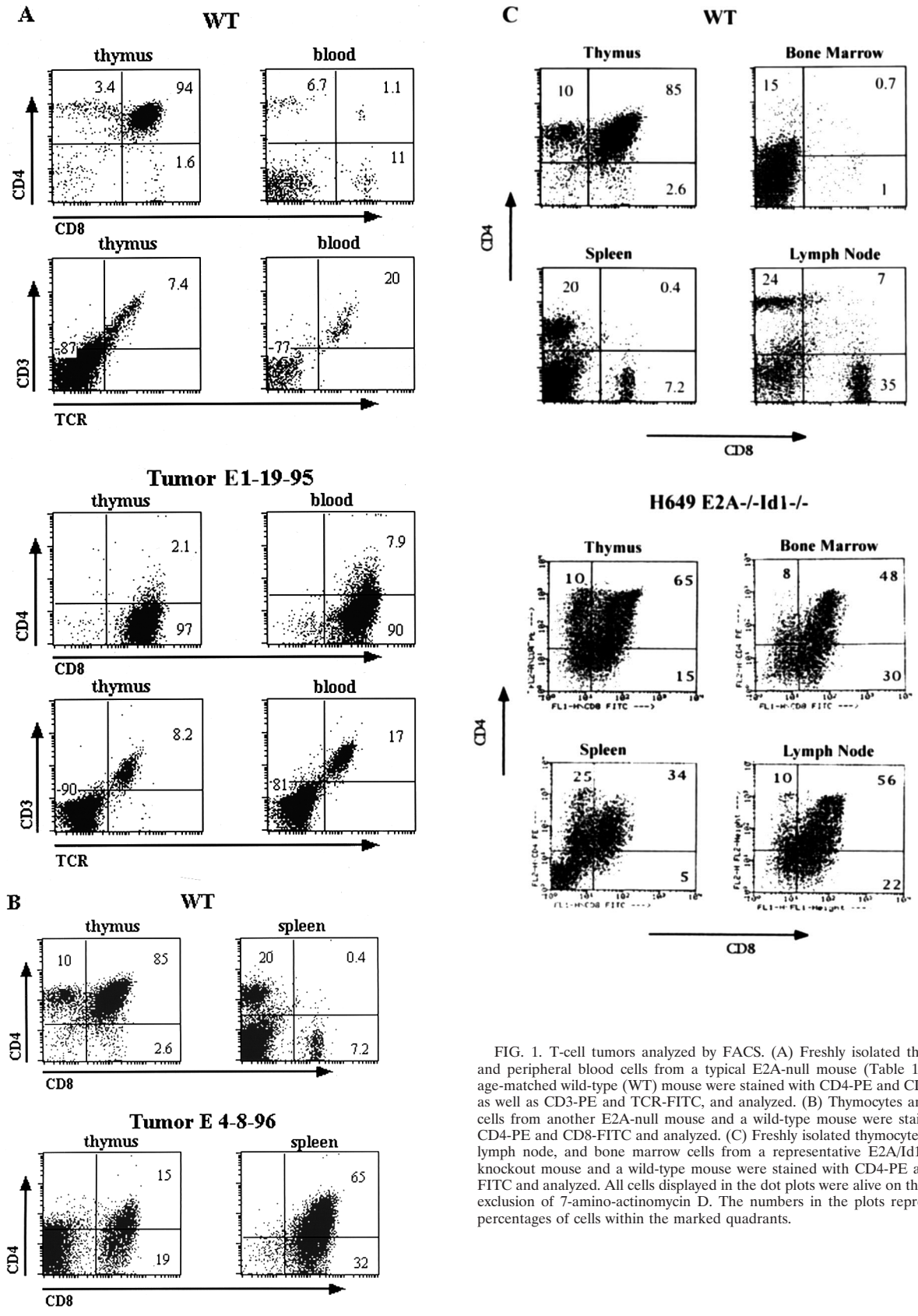

FIG. 1. T-cell tumors analyzed by FACS. (A) Freshly isolated thymocytes and peripheral blood cells from a typical E2A-null mouse (Table 1) and an age-matched wild-type (WT) mouse were stained with CD4-PE and CD8-FITC, as well as CD3-PE and TCR-FITC, and analyzed. (B) Thymocytes and spleen cells from another E2A-null mouse and a wild-type mouse were stained with CD4-PE and CD8-FITC and analyzed. (C) Freshly isolated thymocytes, spleen, lymph node, and bone marrow cells from a representative E2A/Id1 double-knockout mouse and a wild-type mouse were stained with CD4-PE and CD8-FITC and analyzed. All cells displayed in the dot plots were alive on the basis of exclusion of 7-amino-actinomycin D. The numbers in the plots represent the percentages of cells within the marked quadrants.

TABLE 1. Incidence of T-cell tumors in E2A-null mice and E2A/Id1 double-knockout mice[a]

Mouse	Genotype	Age (mo)	Metastasis to lymphoid and nonlymphoid organs[b]	T-cell markers
E 9-19-94	$E2A^{-/-}$	3.5	Yes	NA[c]
E 1-19-95	$E2A^{-/-}$	10	Yes	$CD4^-$ $CD8^+$
E 8-12-95	$E2A^{-/-}$	5	No	$CD4^+$ $CD8^+$ $CD4^-$ $CD8^+$
E 11-12-95	$E2A^{-/-}$	6	No	$CD4^+$ $CD8^+$
E 4-8-96	$E2A^{-/-}$	8	Yes	$CD4^+$ $CD8^+$ $CD4^-$ $CD8^+$
E 9-24-96	$E2A^{-/-}$	5	No	NA
H 206	$E2A^{-/-}$ $Id1^{-/-}$	5	Yes	NA
H 424	$E2A^{-/-}$ $Id1^{-/-}$	3	Yes	$CD4^+$ $CD8^-$
H 547	$E2A^{-/-}$ $Id1^{-/-}$	3.5	Yes	Diffused Mostly $CD4^+$ $CD8^+$
H 643	$E2A^{-/-}$ $Id1^{-/-}$	3.5	Yes	Diffused Mostly $CD4^+$ $CD8^+$
H 649	$E2A^{-/-}$ $Id1^{-/-}$	3.5	No	$CD4^+$ $CD8^+$
H 679	$E2A^{-/-}$ $Id1^{-/-}$	3	Yes	Diffused Mostly $CD4^+$ $CD8^+$

[a] All mice developed thymic lymphomas.
[b] As determined by histological examination and FACS analysis.
[c] NA, not applicable.

readily adapted to growth in tissue culture without addition of cytokines.

It has been shown that E12 and E47 are capable of interacting with the Id1 gene product. It is possible that disruption of the E2A gene leads to an increased level of free Id1 protein which may have deleterious effects, for example, by down-regulating the activities of HEB and E2.2 or other factors. Disruption of Id1 should therefore allow us to determine which phenotypes observed in the E2A knockout mice are attributable to deregulated Id1 activity.

Generation of Id1-null mice. Id1-deficient mice were generated by gene targeting. A null mutation in the Id1 locus was generated by replacing a 0.9-kb genomic fragment encompassing the murine Id1 exon 1 and part of the promoter sequence with a neomycin resistance (*neo*) cassette (Fig. 2A). The disruption of Id1 was confirmed by Southern and Northern analyses (Fig. 2B and C). The $Id1^{-/-}$ mice were born at the expected Mendelian frequency (data not shown) and showed no obvious abnormalities. Both homozygous male and female mice were fertile. Histological analysis upon microscopic examination of brain, muscle, heart, liver, spleen, kidney, adrenal gland, thyroid, gastrointestinal tract, and testis sections from Id1 mutant mice did not reveal any major abnormalities (data not shown). The oldest Id1-null mice are about 2 years old and remain phenotypically normal.

Since Id1 has been implicated in the development of lymphoid and hematopoietic lineages, we examined the cells of these lineages in the Id1-null mice by FACS analysis. Single cell suspensions prepared from the thymus, spleen, bone marrow, and fetal liver were stained with monoclonal antibodies against T-cell markers (CD4, CD8, and CD3, etc.), B-cell markers (B220, CD43, and IgM), myeloid markers (GR-1 and Mac-1), and an erythroid marker (Ter 119). As shown in Fig. 3, no significant differences were found between the Id1 mutant mice and their wild-type littermates.

The lack of phenotype in Id1-null mice suggests the existence of a compensatory mechanism to regulate proliferation and differentiation during development. Interestingly, Id3 knockout mice, recently generated by gene targeting (44a), were also healthy, fertile, and phenotypically normal. Given the fact that Id1 and Id3 have almost identical expression patterns and are biochemically indistinguishable, it is very likely that these two genes share redundant functions in mouse

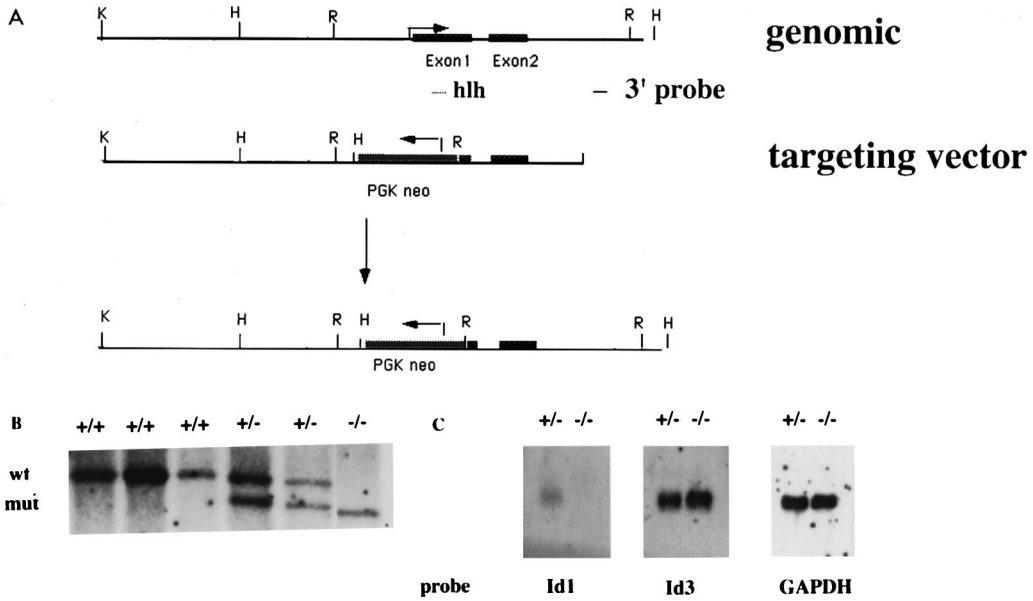

FIG. 2. Targeting of the mouse Id1 locus. (A) Strategy for targeting the mouse Id1 locus. The region of mouse Id1 that was targeted is shown at the top. The targeting vector (middle) contained a *neo* cassette which will replace the first exon and part of the upstream sequences of the Id1 gene. The predicted structure of the targeted Id1 allele is shown at the bottom. The position of the 3′ probe used for Southern analyses is indicated at the top. K, *Kpn*I; H, *Hin*dIII; R, *Eco*RI; PGK, phosphoglycerate kinase. (B) Southern blot of genomic DNA from mice of three Id1 genotypes (+/+, +/−, and −/−). The 3′ probe hybridizes to the 5.0- and 3.5-kb *Eco*RI genomic fragments from the wild-type (wt) and mutant alleles, respectively. (C) Northern blotting performed with RNA isolated from $Id1^{+/-}$ and $Id1^{-/-}$ PEF cells and hybridized with Id1, Id3, and GADPH probes as described in Materials and Methods.

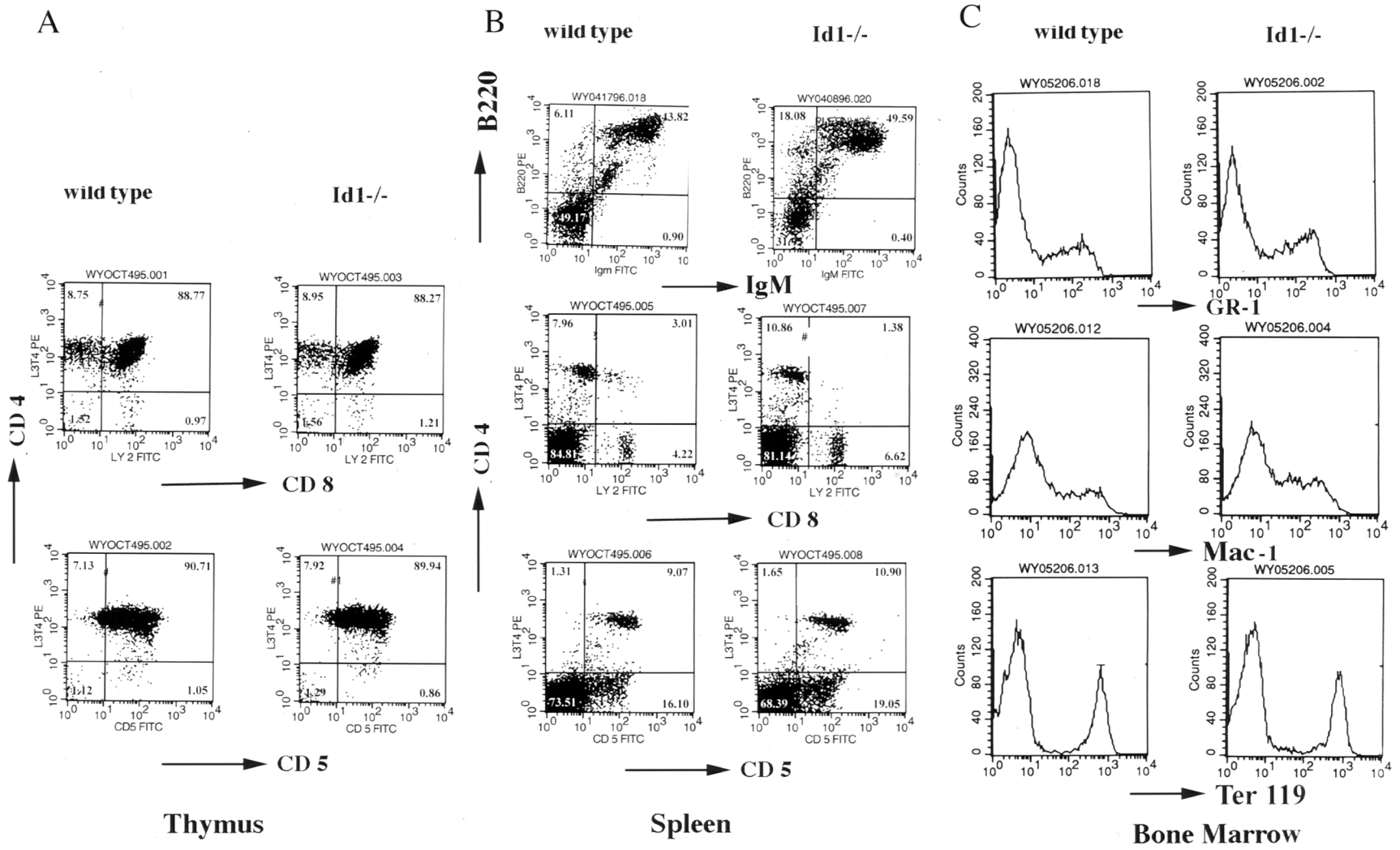

FIG. 3. FACS analysis of hematopoietic cells in Id1 mutant mice. Freshly isolated thymocytes (A), spleen cells (B), and bone marrow cells (C) from Id1 mutant mice and their wild-type littermates were stained with PE- or FITC-conjugated antibodies to various T-cell markers (CD4 and CD8), B-cell markers (B220 and IgM), myeloid markers (Mac-1 and GR-1), and an erythroid marker (Ter 119). The percentage of cells in each quadrant is indicated. No overall differences in total cell numbers were observed in the thymuses, spleens, and bone marrows of Id1-null mice compared to wild-type mice.

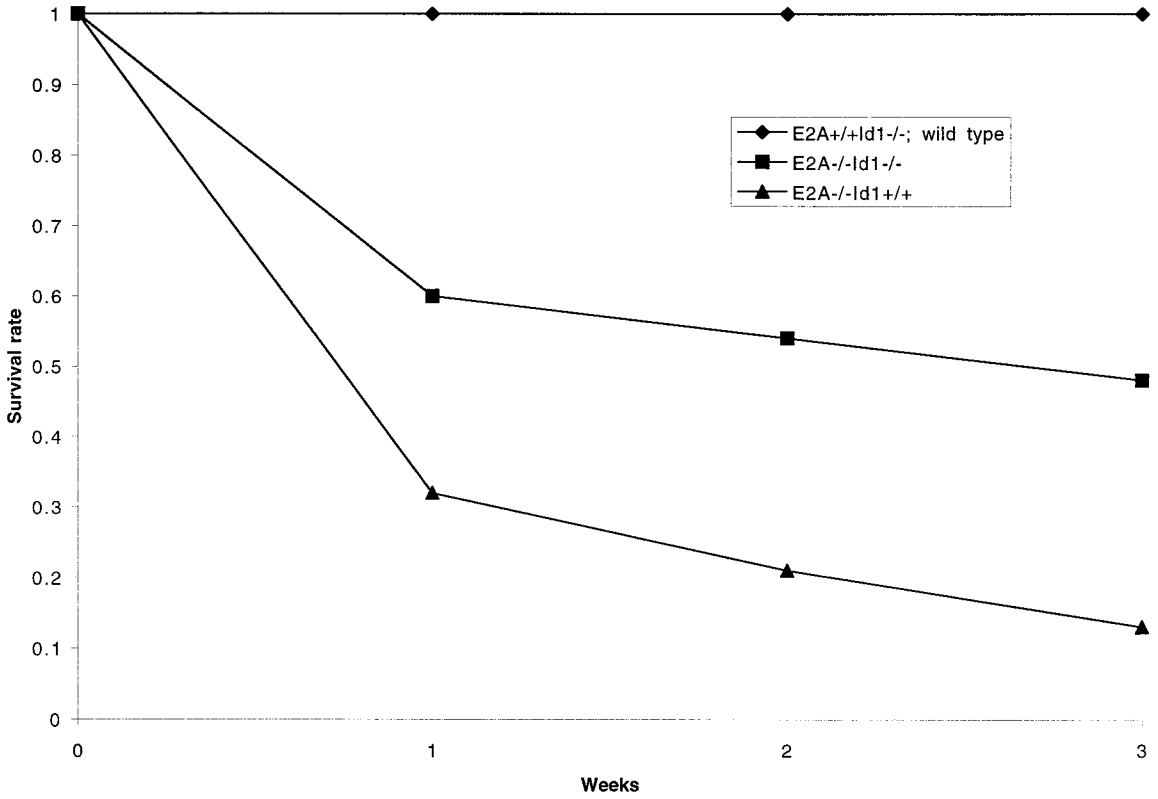

FIG. 4. Survival curve for $E2A^{-/-}$ mice in different Id1 backgrounds. Crosses were set up between $E2A^{+/-}$ male and female mice of the indicated Id1 genotypes. The survival rate for $E2A^{-/-}$ mice generated in each group was calculated based on numbers of $E2A^{-/-}$ mice observed at each time point divided by numbers of $E2A^{-/-}$ mice expected for that particular cross. $E2A^{+/+}$ $Id1^{-/-}$ self-cross was used as a control. The total numbers of $E2A^{-/-}$ mice analyzed in the $Id1^{+/+}$ and $Id1^{-/-}$ background were 22 and 30, respectively. The difference between the survival rate of $E2A^{-/-}$ $Id1^{-/-}$ and $E2A^{-/-}$ $Id1^{+/+}$ mice was significant compared to the difference between that of $E2A^{+/+}$ $Id1^{-/-}$ and $E2A^{+/+}$ $Id1^{+/+}$ mice, as demonstrated by Student's test, with $P < 0.005$. The survival rates of $E2A^{+/+}$ $Id1^{-/-}$ and wild-type mice were identical.

development. This idea has been verified by the analysis of Id1/Id3 double-knockout mice since these mutants are not viable (43a).

Effects of the Id1 mutation on E2A-null mice. To study the effects of the Id1 mutation on E2A-null mice, we set up a genetic cross to generate E2A/Id1 double-mutant mice. Higher numbers of E2A/Id1 double-mutant mice than of E2A-null mice were recorded between the time of birth and 3 weeks of age. Although $E2A^{-/-}$ $Id1^{-/-}$ mice still showed signs of slow growth, their observed survival rate was significantly higher than that of $E2A^{-/-}$ $Id1^{+/+}$ mice before weaning (Fig. 4). Since both the E2A and Id1 knockout mice were bred in the equally mixed background of 129/sv and C57BL/6, the enhancement of survival rate in the double-knockout mice can be explained only by the newly introduced Id1 mutation. This enhancement was not observed with $E2A^{-/-}$ $Id1^{+/-}$ mice (data not shown), most probably indicating a threshold effect of the E2A-to-Id1 ratio.

The partial rescue of $E2A^{-/-}$ neonatal lethality by disruption of the Id1 gene indicates that Id1 is indeed involved in E2A-mediated regulatory pathways at certain stages of development. It is possible that the actual cause of postnatal lethality in E2A-null mice is a decreased E2A/Id ratio, which is elevated in the E2A/Id1 double-knockout mice.

To address the issue of whether the Id1 mutation will rescue the B-cell phenotype in E2A-null mice, we examined B-cell development in E2A/Id1 mice by FACS analysis (Fig. 5). The lymphocyte population in the bone marrow of the double-knockout mice was smaller than that in the $E2A^{+/+}$ $Id1^{-/-}$ mice, and no $B220^{+}$ cells could be detected (Fig. 5B). FACS analysis of embryonic day 18.5 (E18.5) fetal liver detected few, if any, $B220^{dull}$ $CD43^{+}$ cells, indicating that B-cell development in the E2A/Id1 double mutant was blocked at the pro-B-cell stage. The failure of the Id1 mutation to rescue the B-cell defects in E2A-null mice is consistent with the idea that E2A homodimers are essential for B-cell formation and that the loss-of-function E2A mutation cannot be compensated for by increasing the dosage of other E proteins via disruption of Id1.

E2A/Id1 double mutants develop T-cell tumors. All of the first six E2A/Id1 double-knockout mice that survived weaning developed T-cell tumors after 3 months of age. Whether this high rate of tumor formation relative to the single E2A mutant (~50%) remains significant is currently being determined. Nevertheless, the tumors in the double-mutant mice were identical to the tumors found in the E2A-null mice. Necropsy revealed a huge thymic mass in the chest cavity, which may have led to the collapse of the lung and death of the animal (Fig. 6B). Lymphadenopathy was also frequently found in the diseased animals. Histological analysis revealed complete effacement of the normal cortical-medullar structure of the thymus as well as the white pulp and red pulp of the spleen by large immature lymphoblastic cells (Fig. 6D and F). Tumor cells were able to infiltrate peripheral nonlymphoid organs such as the liver, pancreas, and kidney (Fig. 6H, J, and L). FACS analysis showed that the tumor cells express markers

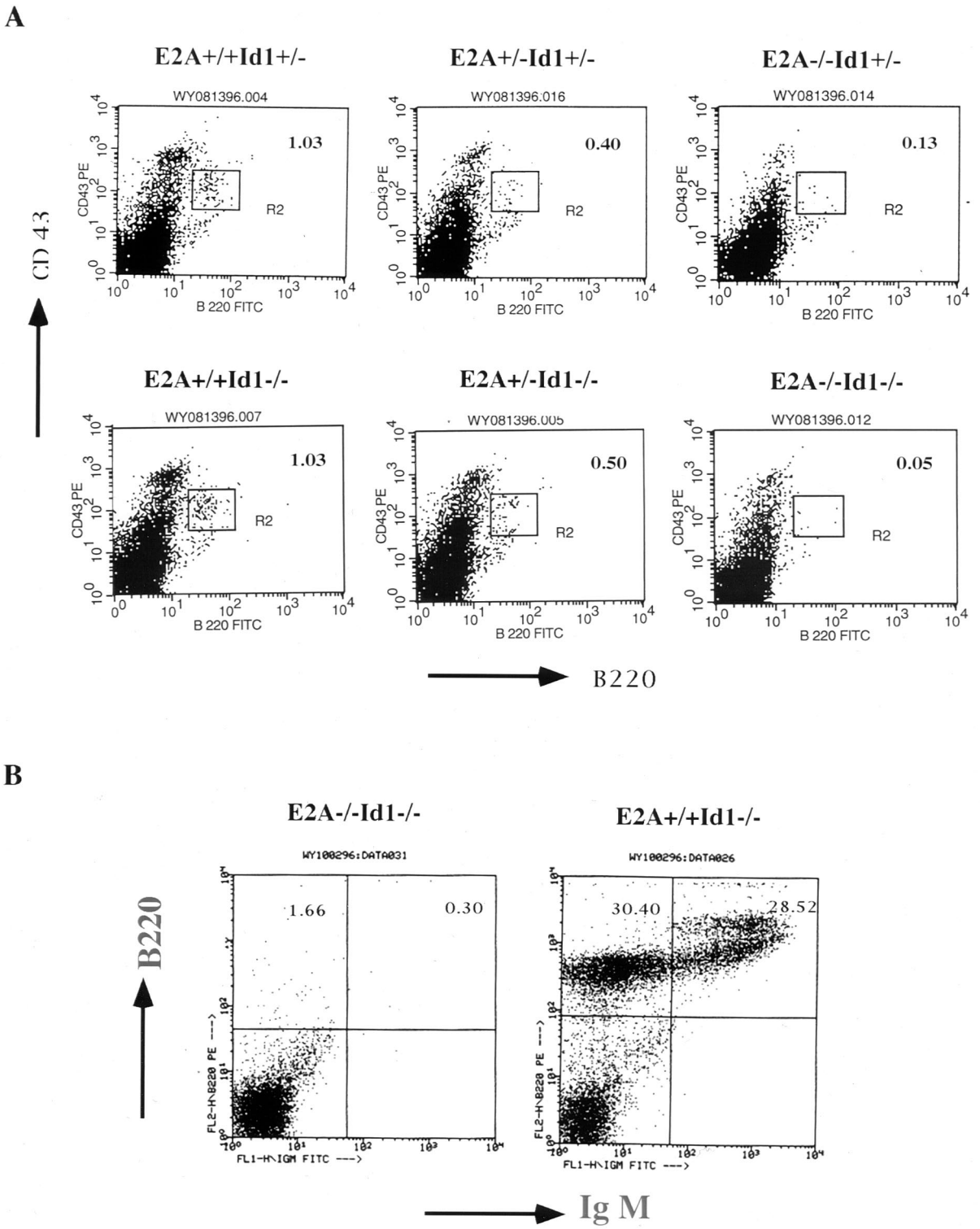

FIG. 5. E2A$^{-/-}$ Id1$^{-/-}$ mice have no B cells (A) Quantitative analysis of pro-B cells in fetal liver of an E2A/Id1 double mutant. The fetal livers from an E18.5 E2A/Id1 double mutant and its littermates were stained with B220-FITC and CD43-PE antibodies and analyzed. B220^{+} CD43^{+} pro-B cells are boxed, and the percentage of cells in each box is indicated. The FACS profile of E18.5 wild-type fetal liver was identical to those of E2A$^{+/+}$ Id1$^{+/-}$ and E2A$^{+/+}$ Id1$^{-/-}$ fetal livers (data not shown). (B) FACS analysis of bone marrow cells from E2A/Id1 double-mutant and E2A$^{+/+}$ Id1$^{-/-}$ mice. Bone marrow cells were stained with B220-PE and IgM-FITC antibodies and analyzed. The numbers in the plots are the percentages of cells within the marked quadrants. The FACS profiles of wild-type and E2A$^{+/+}$ Id1$^{-/-}$ bone marrow cells were identical (data not shown).

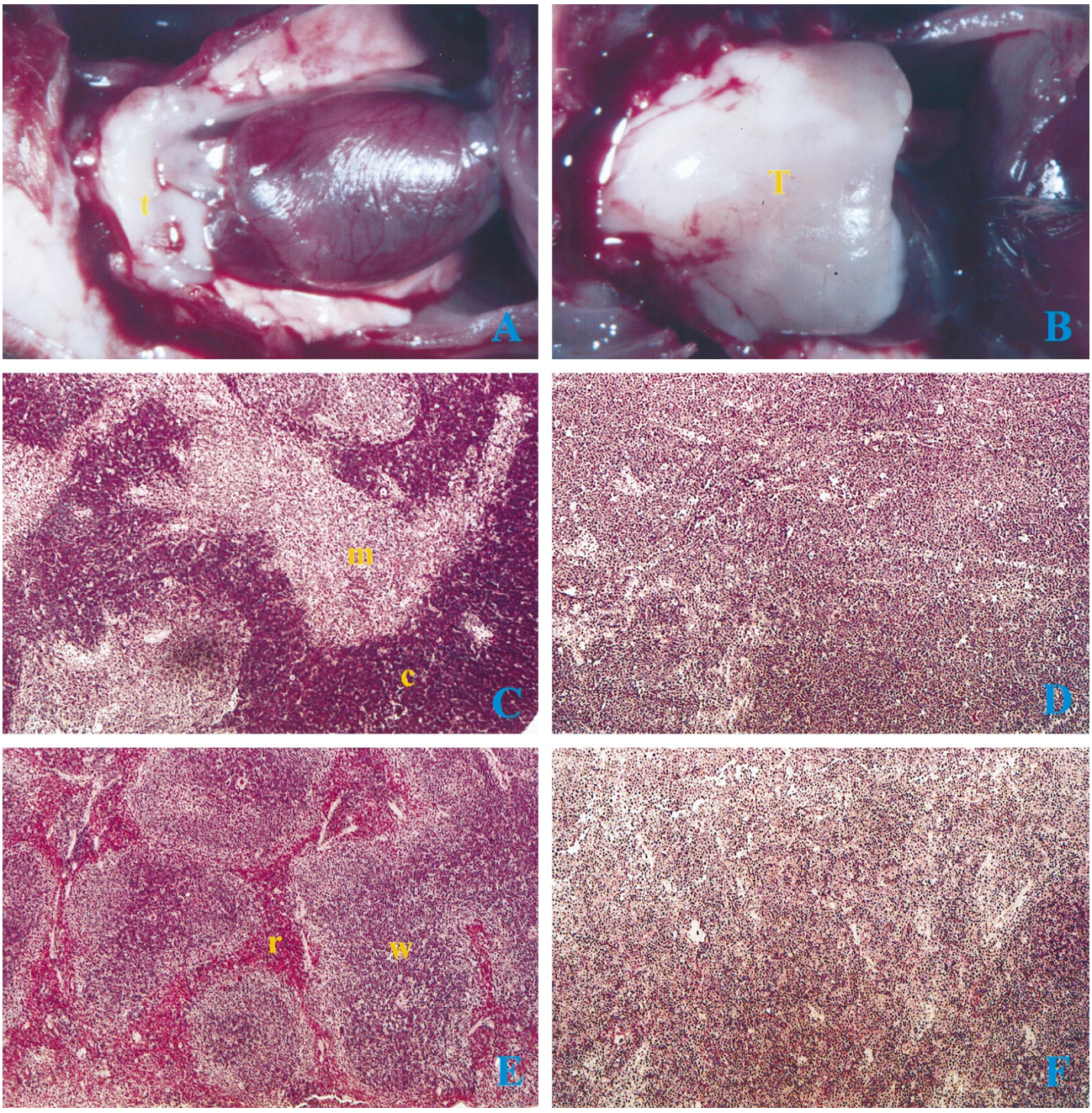

FIG. 6. Morphology of T-cell tumors in E2A-null and E2A/Id1 double-knockout mice compared to age-matched wild-type mice. (A and B) Gross appearance of wild-type thymus (A; t) and thymic lymphoma (B; T) found in 6-month-old E2A/Id1 double-knockout mice. Magnification, ×4.3. (C to F) Hematoxylin-and-eosin-stained tissue sections from thymuses and spleens of 6-month-old wild-type (C and E) and E2A/Id1 double-knockout (D and F) mice. The distinct outer cortex (c) and the inner medulla (m) of the thymus, as well as the white pulp (w) and red pulp (r) of the spleen, were completely replaced by darkly staining lymphocytes in the double mutant. Magnification, ×86. (G to L) Metastasis of lymphoblastic cells to liver, pancreas, and kidney. Hematoxylin-and-eosin-stained tissue sections of livers (G and H), pancreases (I and J), and kidneys (K and L) from wild-type (G, I, and K) and E2A/Id1 double-knockout (H, J, and L) mice. Arrows indicate invading lymphoblasts. Magnification, ×86. T-cell tumors in E2A-null and E2A/Id1 double-knockout mice were identical in morphology (data not shown).

representing various stages of T-cell development (Fig. 1 and Table 1).

The appearance of T-cell tumors in the E2A/Id1 double mutant indicates that the Id1 mutation cannot rescue the tumor phenotype of E2A-null mice. This observation argues against the idea that the T-cell tumors in E2A-null mice are caused by the elevated level of free Id proteins. Instead, it suggests that an inherent function of E2A is primarily responsible for maintaining the homeostasis of T cells during thymocyte development.

DISCUSSION

In this report, we have shown that disruption of the E2A gene results in a high incidence of thymic T-cell tumors. This phenotype indicates that E2A may have, in addition to the

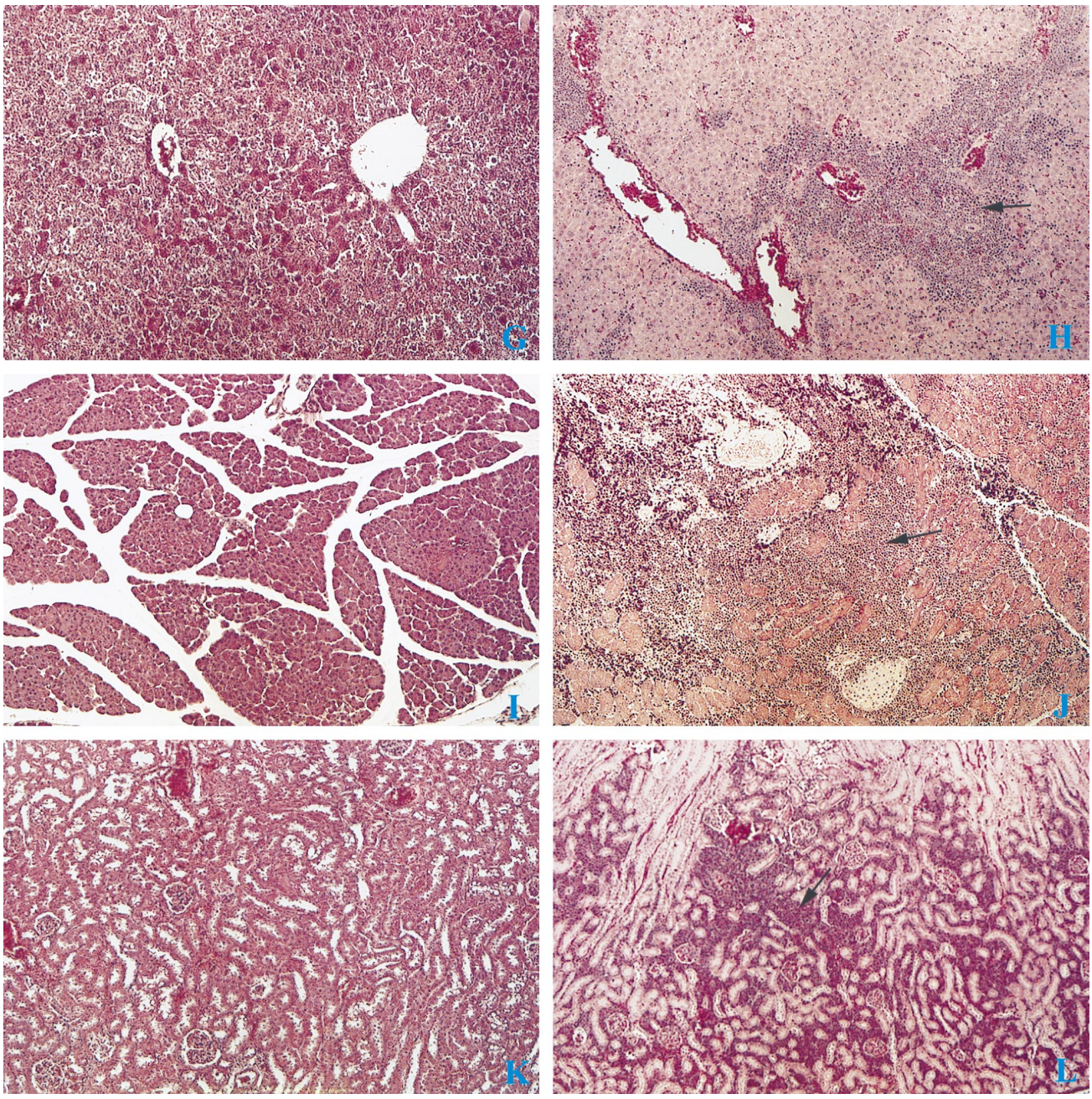

FIG. 6—*Continued.*

previously demonstrated roles in development, a potential tumor-suppressing activity.

In humans, chromosomal translations at the E2A gene locus were shown to be a major cause of childhood lymphocytic B-cell leukemia. In the context of translocations, E2A has oncogenic activity as fusion proteins (9, 21, 42). The targeted E2A allele generated in our experiment still has the potential to produce a truncated molecule that contains the same activation domain found in the oncoproteins. However, this truncated molecule per se is not sufficient to account for the observed T-cell tumors because these tumors were rarely detected in the E2A heterozygotes. We argue that the loss of function of E2A may be the main reason why these mice develop T-cell tumors. This interpretation suggests that the mechanism involved in causing the T-cell tumors in mice lacking the E2A gene may be different from that causing B-ALL in humans.

The tumors detected were only of T-cell origin, suggesting that the oncogenic events may be caused by changes in normal T-cell development programs. Early studies have shown that E2A is required for the proper expression of CD4 and CD5 genes during the early stages of thymocyte development (43). This regulation is apparently achieved in concert with the gene product of HEB. It was found that the amounts of CD4 and CD5 surface antigens were dependent on the combined dosage of both E2A and HEB proteins (43). In the absence of either

E2A or HEB, the cellularity of the thymus is significantly decreased in neonates, suggesting that a developmental block has occurred during T-cell development (44a). However, the blockade is not tight since normal numbers of mature T cells are detected in the peripheral lymph organs of E2A-null or HEB-null animals (43). We reasoned that if the T-cell tumors in the E2A-null mice are triggered by a slowdown in T-cell development, we would expect to see the same or perhaps a higher frequency of T-cell tumors in the adult HEB-null mice. Unfortunately, the neonatal mortality of HEB-null mice is much higher than that of E2A-null mice. After 2 years of breeding of HEB heterozygous mice, only two HEB homozygous mutant mice were found to have survived weaning. Both mice (one is more than 1 year old, and the other is 3 months old) are apparently healthy. Although this limited data set indicates that HEB-null mice can live without developing T-cell tumors, further analysis is still needed before we can fully evaluate the functional relevance of HEB in tumor formation. However, it is also possible that similar to the role of E2A homodimers in B-cell development, the function of E2A in T-cell regulation is unique to E2A itself.

Another possible effect of E2A disruption is the increased level of free Id proteins. The increased free Id proteins may be a potential risk factor in tumorigenesis. Id proteins have been implicated in several tissue culture systems to function as key regulators that inhibit differentiation and promote proliferation. To determine whether any phenotype observed in E2A-null mice is attributable to the increased level of Id proteins, we generated Id1 knockout mice and E2A/Id1 double-knockout mice. Id1 knockout mice were viable and lacked any obvious abnormality. In particular, no obvious defect in the B-cell differentiation pathway was noted. In contrast, overexpression of Id1 in tissue culture models has been shown to antagonize E-protein-mediated activation of Ig enhancers (31, 43). In addition, overexpression of Id1 in the B cells of transgenic mice perturbs B-cell differentiation or causes cell-type-specific killing (38). Whether the failure to observe a phenotype in the B-cell lineage by disruption of Id1 is due to compensation by the other Id genes is currently being investigated.

E2A/Id1 double-knockout mice showed an increased postnatal survival rate compared to E2A-null mice, indicating the possible genetic interaction between the two genes in postnatal regulation. However, the Id1 mutation failed to rescue the B-cell and T-cell phenotypes of E2A-null mice. The E2A/Id1 double-knockout mice still lacked B cells and developed T-cell tumors after 3 months. Our data are consistent with the idea that E2A proteins, rather than their bHLH partners, are primarily responsible for maintaining the homeostasis of T cells during thymocyte development. However, further manipulation of the other three Id genes is required to rule out any contribution of elevated Id activity in the E2A-null mice.

These studies may be helpful in elucidating the mechanism of human T-ALL induced by aberrant expression of the *TAL1* gene. *TAL1* encodes an erythroid-cell-specific bHLH protein which dimerizes with E2A proteins (3, 6, 14). About a quarter of T-ALL patients carry the activated *TAL1* gene in T cells (33). It is not known whether this type of T-ALL is caused by the oncogenic activity of *TAL1* or the ability of *TAL1* to block the normal function of E2A. Interestingly, a transgenic model (23) demonstrated that the onset of T-cell tumors in the *TAL1* transgenic mice could be accelerated by increasing the activity of casein kinase II, an enzyme which can phosphorylate E47 and disrupt E47 homodimer formation (20). While these transgenic studies suggest an involvement of loss of function of E2A in human T-ALL, formal proof is still lacking. The mouse model established in this study may provide a valuable tool for determining the molecular mechanism of human T-ALL diseases.

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REFERENCES

1. **Bain, G., E. C. Maandag, D. J. Izon, D. Amsen, A. M. Kruisbeek, B. C. Weintraub, I. Krop, M. S. Schlissel, A. J. Feeney, M. van Roon, et al.** 1994. E2A proteins are required for proper B cell development and initiation of immunoglobulin gene rearrangements. Cell **79:**885–892.
2. **Barone, M. V., R. Pepperkok, F. A. Peverali, and L. Philipson.** 1994. Id proteins control growth induction in mammalian cells. Proc. Natl. Acad. Sci. USA **91:**4985–4988.
3. **Begley, C. G., P. D. Alplan, S. M. N. Denning, V. F. Haynes, T. A. Waldmann, and I. R. Kirsch.** 1989. The gene SCL is expressed during early hematopoiesis and encodes a differentiation-related DNA-binding motif. Proc. Natl. Acad. Sci. USA **86:**10128–10132.
4. **Benezra, R.** 1994. An intermolecular disulfide bond stabilizes E2A homodimers and is required for DNA binding at physiological temperatures. Cell **79:**1057–1067.
5. **Benezra, R., R. L. Davis, D. Lockshon, D. L. Turner, and H. Weintraub.** 1990. The protein Id: a negative regulator of helix-loop-helix DNA binding proteins. Cell **61:**49–59.
6. **Chen, Q., J. T. Cheng, L. H. Tasi, N. Schneider, G. Buchanan, A. Carroll, W. Crist, B. Ozanne, M. J. Siciliano, and R. Baer.** 1990. The tal gene undergoes chromosome translocation in T cell leukemia and potentially encodes a helix-loop-helix protein. EMBO J. **9:**415–424.
7. **Choi, J. K., C. P. Shen, H. S. Radomska, L. A. Eckhardt, and T. Kadesch.** 1996. E47 activates the Ig-heavy chain and TdT loci in non-B cells. EMBO J. **15:**5014–5021.
8. **Christy, B. A., L. K. Sanders, L. F. Lau, N. G. Copeland, N. A. Jenkins, and D. Nathans.** 1991. An Id-related helix-loop-helix protein encoded by a growth factor-inducible gene. Proc. Natl. Acad. Sci. USA **88:**1815–1819.
9. **Dedera, D. A., E. K. Waller, D. P. LeBrun, A. Sen-Majumdar, M. E. Stevens, G. S. Barsh, and M. L. Cleary.** 1993. Chimeric homeobox gene E2A-PBX1 induces proliferation, apoptosis, and malignant lymphomas in transgenic mice. Cell **74:**833–843.
10. **Ellis, H. M., D. R. Spann, and J. W. Posakony.** 1990. Extramachrochaete, a negative regulator of sensory organ development in Drosophila, defines a new class of helix-loop-helix proteins. Cell **61:**27–38.
11. **Hara, E., J. A. Uzman, G. P. Dimri, J. O. Nehlin, A. Testori, and J. Campisi.** 1996. The helix-loop-helix protein Id-1 and a retinoblastoma protein binding mutant of SV40 T antigen synergize to reactivate DNA synthesis in senescent human fibroblasts. Dev. Genet. **18:**161–172.
12. **Hara, E., T. Yamaguchi, H. Nojima, T. Ide, J. Campisi, H. Okayama, and K. Oda.** 1994. Id-related genes encoding helix-loop-helix proteins are required for G1 progression and are repressed in senescent human fibroblasts. J. Biol. Chem. **269:**2139–2145.
13. **Henthorn, P., M. Kiledjian, and T. Kadesch.** 1990. Two distinct transcription factors that bind the immunoglobulin mE5/kE2 motif. Science **247:**467–470.
14. **Hsu, H. L., I. Wadman, and R. Baer.** 1994. Formation of in vivo complexes between the TAL1 and E2A polypeptides of leukemic T cells. Proc. Natl. Acad. Sci. USA **91:**3181–3185.
15. **Hu, J. S., E. N. Olson, and R. E. Kingston.** 1992. HEB, a helix-loop-helix protein related to E2A and ITF2 that can modulate the DNA-binding ability of myogenic regulatory factors. Mol. Cell. Biol. **12:**1031–1042.
16. **Inaba, T., W. M. Roberts, L. H. Shapiro, K. W. Jolly, S. C. Raimondi, S. D. Smith, and A. T. Look.** 1992. Fusion of the leucine zipper gene HLF to the E2A gene in human acute B-lineage leukemia. Science **257:**531–534.
17. **Jan, Y. N., and L. Y. Jan.** 1993. HLH proteins, fly neurogenesis, and vertebrate myogenesis. Cell **75:**827–830.
18. **Jen, Y., K. Manova, and R. Benezra.** 1996. Expression patterns of Id1, Id2, and Id3 are highly related but distinct from that of Id4 during mouse embryogenesis. Dev. Dyn. **207:**235–252.
19. **Jen, Y., H. Weintraub, and R. Benezra.** 1992. Overexpression of Id protein inhibits the muscle differentiation program: in vivo association of Id with E2A protein. Genes Dev. **6:**1466–1479.
20. **Johnson, S. E., X. Wang, S. Hardy, E. J. Taparowsky, and S. F. Konieczny.** 1996. Casein kinase II increases the transcriptional activities of MRF4 and MyoD independently of their direct phosphorylation. Mol. Cell. Biol. **16:**1604–1613.
21. **Kamps, M. P., and D. Baltimore.** 1993. E2A-Pbx1, the t(1;19) translocation

protein of human pre-B-cell acute lymphocytic leukemia, causes acute myeloid leukemia in mice. Mol. Cell. Biol. **13:**351–357.
22. **Kamps, M. P., C. Murre, X. H. Sun, and D. Baltimore.** 1990. A new homeobox gene contributes the DNA binding domain of the t(1;19) translocation protein in pre-B ALL. Cell **60:**547–555.
23. **Kelliher, M. A., D. C. Seldin, and P. Leder.** 1996. Tal-1 induces T cell acute lymphoblastic leukemia accelerated by casein kinase IIalpha. EMBO J. **15:** 5160–5166.
24. **Kreider, B., R. Benezra, G. Roverea, and T. Kadesch.** 1992. Inhibition of myeloid differentiation by the helix-loop-helix protein Id. Science **255:**1700–1702.
25. **Lassar, A. B., R. L. Davis, W. E. Wright, T. Kadesch, C. Murre, A. Varonova, D. Baltimore, and H. Weintraub.** 1991. Functional activity of myogenic HLH proteins requires hetero-oligomerization with E12/E47-like proteins in vivo. Cell **66:**305–315.
26. **Lister, J., W. C. Forrester, and M. H. Baron.** 1995. Inhibition of an erythroid differentiation switch by the helix-loop- helix protein Id1. J. Biol. Chem. **270:**17939–17946.
27. **Murre, C., P. S. McCaw, and D. Baltimore.** 1989. A new DNA binding and dimerization motif in immunoglobulin enhancer binding, daughterless, MyoD and myc proteins. Cell **56:**777–783.
28. **Nourse, J., J. D. Mellentin, N. Galili, J. Wilkinson, E. Stanbridge, S. D. Smith, and M. L. Cleary.** 1990. Chromosomal translocation t(1;19) results in synthesis of a homeobox fusion mRNA that codes for a potential chimeric transcription factor. Cell **60:**535–545.
29. **Olson, E. N., and W. H. Klein.** 1994. bHLH factors in muscle development: dead lines and commitments, what to leave in and what to leave out. Genes Dev. **8:**1–8.
30. **Peverali, F. A., T. Ramqvist, R. Saffrich, R. Pepperkok, M. V. Barone, and L. Philipson.** 1994. Regulation of G1 progression by E2A and Id helix-loop-helix proteins. EMBO J. **13:**4291–4301.
31. **Pongubala, J. M., and M. L. Atchinson.** 1991. Functional characterization of the developmentally controlled immunoglobulin kappa 3′ enhancer: regulation by Id, a repressor of helix-loop-helix transcription factors. Mol. Cell. Biol. **11:**1040–1047.
32. **Riechmann, V., I. van Cruchten, and F. Sablitzky.** 1994. The expression pattern of Id4, a novel dominant negative helix-loop-helix protein, is distinct from Id1, Id2 and Id3. Nucleic Acids Res. **22:**749–755.
33. **Robb, L., and C. G. Begley.** 1996. The helix-loop-helix gene SCL: implicated in T-cell acute lymphoblastic leukaemia and in normal haematopoietic development. Int. J. Biochem. Cell Biol. **28:**609–618.
34. **Schlissel, M., A. Voronova, and D. Baltimore.** 1991. Helix-loop-helix transcription factor E47 activates germ-line immunoglobulin heavy-chain gene transcription and rearrangement in a pre-T-cell line. Genes Dev. **5:**1367–1376.
35. **Shen, C. P., and T. Kadesch.** 1995. B-cell-specific DNA binding by an E47 homodimer. Mol. Cell. Biol. **15:**4518–4524.
36. **Shivdasani, R. A., E. L. Mayer, and S. H. Orkin.** 1995. Absence of blood formation in mice lacking the T-cell leukaemia oncoprotein tal-1/SCL. Nature **373:**432–434.
37. **Sloan, S. R., C. P. Shen, R. McCarrick-Walmsley, and T. Kadesch.** 1996. Phosphorylation of E47 as a potential determinant of B-cell-specific activity. Mol. Cell. Biol. **16:**6900–6908.
38. **Sun, X. H.** 1994. Constitutive expression of the Id1 gene impairs mouse B cell development. Cell **79:**893–900.
39. **Sun, X. H., N. A. Copeland, N. A. Jenkins, and D. Baltimore.** 1991. Id proteins Id1 and 1d2 selectively inhibit DNA binding by one class of helix-loop-helix proteins. Mol. Cell. Biol. **11:**5603–5611.
40. **Swiatek, P. J., and T. Gridley.** 1993. Perinatal lethality and defects in hindbrain development in mice homozygous for a targeted mutation of the zinc finger gene Krox20. Genes Dev. **7:**2071–2084.
41. **Tournay, O., and R. Benezra.** 1996. Transcription of the dominant negative helix-loop-helix protein Id1 is regulated by a protein complex containing the immediate-early response gene *Egr*-1. Mol. Cell. Biol. **16:**2418–2430.
42. **Weintraub, H.** 1993. The MyoD family and myogenesis: redundancy, networks, and thresholds. Cell **75:**1241–1244.
43. **Wilson, R. B., M. Kiledjian, C. P. Shen, R. Benezra, P. Zwollo, S. M. Dymecki, S. V. Desiderio, and T. Kadesch.** 1991. Repression of immunoglobulin enhancers by the helix-loop-helix protein Id: implications for B lymphoid development. Mol. Cell. Biol. **11:**6185–6191.
43a. **Yan, W., and R. Benezra.** Unpublished data.
44. **Yoshihara, T., T. Inaba, L. H. Shapiro, J. Y. Kato, and A. T. Look.** 1995. E2A-HLF-mediated cell transformation requires both the *trans*-activation domains of E2A and the leucine zipper dimerization domain of HLF. Mol. Cell. Biol. **1555:**3247–3255.
44a. **Zhuang, Y.** Unpublished data.
45. **Zhuang, Y., P. Cheng, and H. Weintraub.** 1996. B-lymphocyte development is regulated by the combined dosage of three basic helix-loop-helix genes, E2A, E2-2, and HEB. Mol. Cell. Biol. **16:**2898–2905.
46. **Zhuang, Y., P. Soriano, and H. Weintraub.** 1994. The helix-loop-helix gene E2A is required for B cell formation. Cell **79:**875–884.